

**Male wing color properties predict the size of nuptial gifts given during mating in the
Pipevine Swallowtail butterfly (*Battus philenor*)**

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Abstract In many animals, males bear bright ornamental color patches that may signal both the direct and indirect benefits that a female might accrue from mating with him. Here we test whether male coloration in the Pipevine Swallowtail butterfly, *Battus philenor*, predicts two potential direct benefits for females, copulation duration and the quantity of materials the male passes to the female during mating. In this species, males have a bright iridescent blue field on the dorsal hindwing surface while females have little or no dorsal iridescence. Females preferentially mate with males who display a bright and highly chromatic blue on their dorsal hindwing. In this study, we show that the chroma of the blue on the male dorsal hindwing and male body size (forewing length) significantly predict the mass of material or spermatophore that a male forms within the female's copulatory sac during mating. We also found that spermatophore mass correlated negatively with copulation duration, but that color variables did not significantly predict this potential direct benefit. These results suggest that females may enhance the material benefits they receive during mating by mating with males based on the coloration of their dorsal hindwing.

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Introduction

In many animals, males display elaborate secondary sexual characteristics that females do not have. Many of these ornaments are an evolutionary product of sexual selection in the context of either female mate choice or male-male competition for mates (Andersson 1994). When females display a preference for ornamented males, the honest-signaling or indicator hypothesis suggests that there are two broad classes of benefits for females that may drive the evolution of the preference (Andersson 1994). If the development of the ornament is correlated with male genetic quality, females may gain indirect benefits in the form of high quality genes for their offspring. Alternatively, male ornaments may indicate direct benefits that females and their offspring may receive, such as superior parental care, disease avoidance, or other material aid the male may offer.

Butterflies offer practical and interesting opportunities to examine these potential relationships between male ornament characteristics and the benefits females may gain by mating with highly ornamented males. In many butterflies, males display color patterns that are brighter, more chromatic, and more boldly patterned than those of females. Recent studies suggest that females preferentially mate with males with such color features (for review, Kemp and Rutowski 2011), as Darwin (1871) suggested, and that, because in some species male coloration is heritable, there may be indirect benefits for females in choosing colorful males (Kemp and Rutowski 2007; Kemp 2008).

The evidence that male color ornaments signal potential direct benefits in butterflies is more equivocal. During mating in butterflies, a male forms a package of sperm and nutrients in the female's reproductive tract called a spermatophore. The contents of a spermatophore may provide females with direct benefits in the form of nutrients that she can use in egg production and in her own somatic maintenance to enhance her reproductive output (Boggs and Gilbert 1979; Rutowski et al. 1987; Watanabe and Sato 1993; for a recent review, see South and Lewis 2011). This suggests that females might receive enhanced material benefits from mating with males with features that suggest they will produce a large spermatophore during mating. Such features could include a male's body size which in the Lepidoptera is often correlated with the size of the spermatophore he is likely to produce (Bissondath and Wiklund 1995; Hughes et al. 2000; Lewis and Wedell 2007). A male's coloration is another potential indicator trait. However, Kemp et al. (2008) found that in the Orange Sulphur butterfly, male UV coloration, which is used by females in mate choice, is not correlated with the quality of the spermatophore she is likely to receive from the male during mating.

Nonetheless, in other species, color might be an indicator of the material benefits a male can give a female during mating. For example, coloration degrades with age and wing wear (Kemp 2006), so bright coloration may indicate a low probability of previous mating and the large size or high quality of a spermatophore a young male is likely to produce during copulation. Empirical studies show that age and prior mating history both may negatively influence the size of the spermatophore a male produces during mating (Svard and Wiklund 1986; Rutowski et al. 1987; Oberhauser 1988). Also, females that mate with recently mated males not only receive smaller spermatophores but also endure longer copulation durations and decreased reproductive output (Rutowski et al. 1987; Hughes et al. 2000).

Here we report on the relationship between a male's coloration and two measures of the potential direct benefits of mate choice, spermatophore size and copulation duration, in the Pipevine Swallowtail, *Battus philenor*. Males of this butterfly species display blue iridescent

patches on the ventral and dorsal surfaces (Fig. 1) that serve various signal functions (Rutowski et al. 2010). The ventral iridescent areas are recognized by predators as a warning signal (Brower 1958; Codella and Lederhouse 1990; Pegram et al. 2013) while the dorsal iridescent coloration is an intersexual signal. Dorsal iridescent patches are brighter and more chromatic in males than in females, and females prefer to mate with males with a more chromatic dorsal iridescence (Rutowski and Rajyaguru 2013). Here we test the prediction that male iridescent dorsal coloration generally, and specifically the chroma of that iridescence, is positively correlated with the size of the spermatophore he is likely to produce during mating and negatively correlated with the duration of the copulation.

Materials and methods

Source of animals

Eggs and larvae of *B. philenor* were collected from the area surrounding the confluence of Mesquite Wash and Sycamore Creek (33.2° N, 111.7° W) in the Mazatzal Mountains of central Arizona during the summer months of 2008 and 2009. Larvae and pupae were reared in an environmental chamber (for details see Rutowski et al. 2010). All larvae were fed *ad libitum* on cuttings from their local host plant, *Aristolochia watsonii*. Upon eclosion, animals were stored in a refrigerator at 4°C until use.

Matings

Virgin females that had been refrigerated for no more than 4 days (average = 0.78 days) were taken to the field site described above. Each female was tethered by tying one end of a 0.5m long piece of thread around the base of her abdomen where it narrowly joins the thorax. The other end

of the tether was tied to the end of a 1 m long stick. Each tethered female was presented to free-flying males until one of the males courted and mated with the female. After copulation began, we placed the mating pair in a 500 ml covered cup and checked them regularly until they separated, at which time we recorded the duration of the copulation and placed both animals in a freezer to euthanize and store them until the measurements described below were made.

Body size, male age, and male color assessments

We used forewing length and body mass as indicators of body size for males and females. Forewing length was measured with digital calipers to the nearest 0.1 mm from where the costal vein inserts into the thorax to the tip of the wing. Body mass was measured to the nearest 0.1 mg on an analytical balance after the butterflies were dried under vacuum for 24 hr. The abdomen of each female was dissected under Ringer's solution and her bursa copulatrix removed. We measured the dry mass of each bursa and its contents after 24 hrs of drying under vacuum to remove water. Previous work indicated that immediately after mating in butterflies the mass of a bursa is negligible relative to the mass of the material in it received from the male (Marshall 1980).

After making these measurements we removed the hindwings from males and females and mounted them on black cardboard using 3M photo mount adhesive. The left hindwing was mounted ventral side up and the right hindwing was mounted dorsal side up. For males, we assessed wing wear as a surrogate for age. Wear was scored on a scale of 1 to 5 using the following criteria: 1, no evident wing area or scale loss or tattering of wing edges; 3, moderate wing area and scale loss and tattering of wing edges; 5, extensive loss of wing area and scale, and tattering of wing edges.

We collected reflectance spectra from both the dorsal and ventral iridescent patches of the male hindwings using techniques described in detail in Rutowski et al. (2010). Reflectance

relative to a magnesium oxide white standard was measured between 300 and 700 nm from the regions on the wings shown in Fig. 1. Using the software, CLR (Montgomerie 2008) the following color parameters were extracted from each spectrum after binning the data into 1nm bins.

- 1) Hue: the wavelength (nm) at which percent reflectance relative to the white standard was greatest
- 2) Chroma: the percent of the total reflectance from 300 – 700 nm found within a wavelength segment spanning 50 nm on either side of the wavelength of peak reflectance
- 3) Brightness: the average percent reflectance in wavelengths from 300-700 nm

Statistical Analysis

To determine which phenotypic variables best predicted spermatophore mass and copulation duration, we ran two stepwise linear regressions with spermatophore mass and copulation duration as the dependent variables, respectively. Some of our predictor variables were correlated and so to reduce the effects of multicollinearity, we first evaluated the strength of these correlations using a Pearson correlation analysis with a two-tailed test. For any pair of significantly correlated variables, we removed one of the variables in the two ensuing regression analyses (see list of variables included in each model and explanation in the Results). This was done in lieu of a principal components analysis to facilitate interpretation of the results. We used SPSS v. 21 (IBM, Armonk, NY) and a 0.05 level of significance for all statistical analyses.

Results

The data collected from 75 mated pairs are summarized in Table 1. There was substantial variation for our dependent variables, spermatophore mass and copulation duration, both ranging over almost an order of magnitude.

The correlation analysis on our independent variables revealed several significant correlations that were taken into consideration in our decisions about which variables to include in the regression analysis. First, there were strong correlations between forewing length and body mass (males: $r = 0.84$, $p < 0.001$; females: $r = 0.771$, $p < 0.001$). We used only forewing length in subsequent analyses because this measure does not change with an individual's age and adult history whereas mass is expected to decrease with age as indicated by wing wear, as it did at least for males ($r = -0.25$, $p = 0.03$).

All ventral hindwing color parameters as well as dorsal hindwing brightness were also omitted from the independent variables for several reasons. First, experimental studies suggested that females attend to male dorsal and not ventral hindwing coloration and so correlations between ventral coloration and our dependent variables were not of interest (Rutowski and Rajyaguru 2013). Second, several correlations between ventral and dorsal hindwing color parameters suggested this was appropriate. From prior studies (Rutowski et al. 2010) and in this data set, there were significant positive correlations between dorsal and ventral hindwing hue ($r = 0.63$, $p < 0.001$) hence we included only dorsal hindwing hue in the regression analysis. Also, we did not include any measure of hindwing brightness in the analysis for two reasons. First, previous experiments indicated that it was not correlated with male mating success (Rutowski and Rajyaguru 2013) and dorsal brightness was negatively correlated with dorsal chroma ($r = -0.506$, $p < 0.001$) on the hindwing. Finally, wing wear was included in the analysis but ventral hindwing chroma was not because it was negatively correlated with wing wear ($r = -0.432$, $p < 0.001$).

Again, these exclusions were done to control confounding covariation among independent variables and facilitate interpretation of the results. The final multiple regression model included these independent variables: male forewing length, female forewing length, male wing wear, male dorsal hindwing chroma, and male dorsal hindwing hue. In a stepwise multiple regression this suite of variables significantly predicted spermatophore mass (ANOVA, $p = 0.004$) but did not predict copulation duration (ANOVA, $p = 0.453$). Moreover, the only variables that contributed significantly to the prediction of spermatophore mass were (1) male forewing length ($p = 0.014$), i.e., larger males produced larger spermatophores, and (2) dorsal hindwing chroma ($p = 0.045$), i.e., more chromatic males produced larger spermatophores (Fig. 2).

Although no independent variable included in our regression model predicted copulation duration we did find that the mass of the spermatophore produced in copulation was negatively correlated with the duration of copulation (Fig. 3).

Discussion

The results help identify those features of the participants in a mating that may influence the size of the spermatophore the female receives. Larger males produce larger spermatophores which is consistent with prior reports for *B. philenor* (Rutowski et al. 1989) and other butterfly species (Rutowski 1984; Rutowski and Gilchrist 1986, 1987; Svärd and Wiklund 1986; Bissoondath and Wiklund 1996; Wedell and Cook 1999; Hughes et al. 2000) and may reflect that larger males have more resources to commit to the production of a spermatophore and its contents. We found not relationship between female body size and the size of the spermatophore she receives Which agrees with results from other butterfly species (Bissoondath and Wiklund 1996; Hughes et al. 2000; Rutowski 1984).

Direct benefits and male coloration

The chroma of a dorsal male's hindwing coloration was the only color variable that regression analysis revealed to be a significant predictor of the quantity of material he placed in the female during mating. This relationship was positive, i.e., more chromatic males produced on average larger spermatophores, as we expected given the results of our mate choice experiments in which the males with the highest mating success were those that were most chromatic (Rutowski and Rajyaguru 2013). This supports the hypothesis that females are choosing among males on the basis of the properties of an ornament in a way that will maximize the direct benefits they accrue from the mating. This is one of only a few studies showing that a known sexual signal that is important in mate choice may reliably predict the nuptial gift a female is likely to receive from a male in species in which the quality of the nuptial gift cannot be directly evaluated (Dussourd et al. 1991; Lewis and Cratsley 2008).

Brief copulation duration is another potential direct benefit. In butterflies, males control the duration of copulation (Wickman 1985) and mating pairs are perhaps exposed to greater risk of predation, and so the briefer the copulation better. However, in this study no color parameter included in the analysis was a significant predictor of copulation duration, including dorsal hindwing chroma.

We emphasize that male dorsal coloration is most likely an indicator of his overall potential to produce material benefits and not his recent mating history. A male's previous mating history affects the mass of the spermatophore he can produce at a given time in his life (Bissoondath and Wiklund 1996; Hughes et al. 2000) which can have fitness consequences for females (Rutowski et al. 1987, Svard and Wiklund 1991). Replenishment of the materials available to put into spermatophores can take several days in *B. philenor* (Rutowski et al. 1989). However, recent mating history cannot in butterflies lead to changes in a male's color signal

which is set at eclosion and in *B. philenor* surprisingly changes little, if at all, with age (Rutowski et al. 2010).

Iridescent reflections as signals

The iridescent properties of the male's blue coloration mean that because of the changes in the relative positions of the female and the male's wings during courtship, the perceived color of the male's dorsal wing surface may change dramatically over a range of wavelengths (Rutowski et al. 2010). For a given position of receiver and light source above a wing surface, both the brightness and the chroma of the reflection seen by the viewer will change as the wing moves during a wing beat cycle. Hue might also vary if the light source that contributes to a visible reflection can come from multiple directions above the wing surface, i.e. from different points in the blue sky and so arrive at different angles of incidence. These effects are expected to make it difficult for a receiver to assess reliably the relative chromaticity or brightness, and hue of an individual male's reflection relative to some threshold or internalized standard. This problem could be reduced if the relative positions of male and female are somehow "standardized" during courtship. For some birds that use iridescent color signals, signaler behavior does appear to be structured to maximize the transmission of their iridescent signal to the intended receiver (Hamilton 1965; Loyau et al. 2007). In sulphur butterflies, males position themselves relative to conspecifics in ways that enhance their ability to assess whether or not an approached conspecific has an iridescent UV reflection (Rutowski et al 2007). Those that do not, namely, females, are courted. High speed video recordings of the behavior of *B. philenor* males and females in courtship that are currently being analyzed in our lab suggest this is the case (unpubl. data).

Is male dorsal hindwing iridescence costly?

Our results with *B. philenor* provide evidence that female color preference may be adaptive in that it maximizes the size of the nuptial gift she receives during mating. However, for such an indicator signal to evolve, it requires that there be costs associated with its production that prevent males from cheating, that is, developing a colorful ornament even when they are not able to produce the costly contents of a spermatophore. During development, the photonic structures that produce iridescent colors in animals may be especially costly to build because of the precision required in nanoscale construction to produce a bright and chromatic iridescent reflection (McGraw et al. 2002, Kemp 2006, Kemp and Macedonia 2006, Kemp and Rutowski 2007) and therefore exhibit condition dependence (e.g. Doucet et al. 2006, Kemp 2006, Kemp and Rutowski 2007). Pegram et al. (in press) have examined which features of the coloration of *B. philenor* are affected by food deprivation. Food deprivation had negative effects on body size, a measure of condition, in that study and so offered an indicator of whether color features, especially dorsal hindwing chroma are costly to produce (Cotton et al. 2004). Contrary to expectation, chroma was not affected by food deprivation which leaves open the question of whether chroma could evolve as a reliable indicator trait.

However, there are two features of the study by Pegram et al. (in press) that leave this question open. First, the effect of food deprivation was evaluated under only a single set of controlled growth conditions. Perhaps there are significant effects of food deprivation on chroma under other regimes of temperature and humidity in the highly variable field environment. Second, only a single stressor was examined. There are other possible stressors such as disease, extreme environmental variation, and foodplant quality that might affect chroma as well as a male's ability to produce a spermatophore (Kemp and Rutowski 2007). These possibilities are supported by the fact that the chroma of the male's dorsal hindwing of lab-reared *B. philenor* is different from that of field caught-individuals, a result not fully explained by higher levels of wing wear in the field-caught individuals (Rutowski et al. 2010). Wing wear has no significant effect on dorsal hindwing coloration in this species.

There also remains the possibility that females benefit from selecting chromatic males for indirect rather than direct benefits. The intrasexual variation in male coloration has a genetic basis in other butterflies (Kemp and Rutowski 2007, Kemp 2008). For *B. philenor*, the details of the proximate causes of naturally-occurring variation in male dorsal hindwing coloration (especially chroma) and ability to produce a spermatophore, and the consequences of this variation for female reproductive success, warrant further investigation and will inform our understanding of the evolution of this female color preference in this and other species.

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Table 1 Descriptive statistics for the measured variables (n = 75). See text for details on measurements, especially wing wear, brightness, and chroma.

Variable	mean	SD	minimum-maximum
Copulation Duration (min)	147	88.1	52-507
Spermatophore dry mass (mg)	6.5	2.02	2.1-10.8
Female: Forewing length (mm)	49	3.1	39-55
Body mass (mg)	398	87	194-550
Male: Forewing length (mm)	42	3.3	32-48
Body mass (mg)	178	49.5	81-327
Wing wear	2.7	1.09	1-5
Dorsal iridescent patch			
Brightness	9.3	3.6	2.7-22.1
Hue (nm)	489	16.6	441-533
Chroma	0.501	0.057	0.392-0.717
Ventral iridescent patch			
Brightness	35.5	10.7	18.7-77
Hue (nm)	490	21.3	451-566
Chroma	0.428	0.025	0.322-0.475



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422 Figure 1. The dorsal (left) and ventral (right) wing surfaces of a *B. philenor* male photographed
423 under conditions that maximize the visibility of the iridescent blue to the camera. The red circles
424 show the region on each wing surface from which reflectance measurements were taken.

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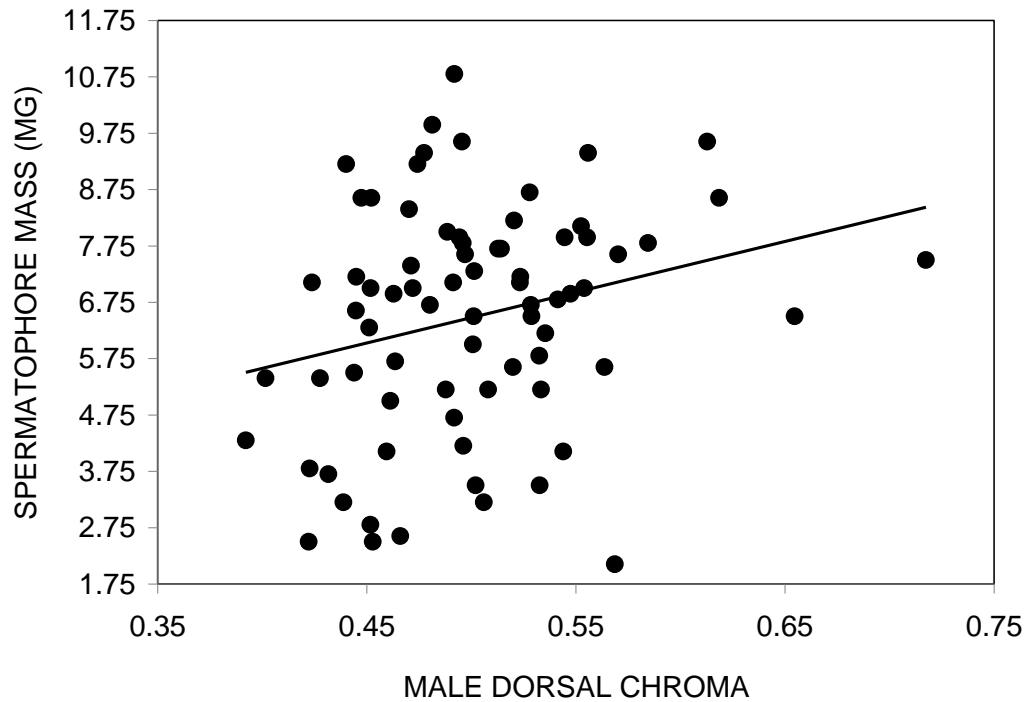


Figure 2. The relationship between the mass of the spermatophore a male produces and the chroma of the iridescent area on his dorsal hindwing surface. The line is the linear best fit from a simple regression ($r = 0.253$, 74 df, $p < 0.029$). The multiple regression analysis also supported chroma as a predictor of spermatophore mass (see text for details).

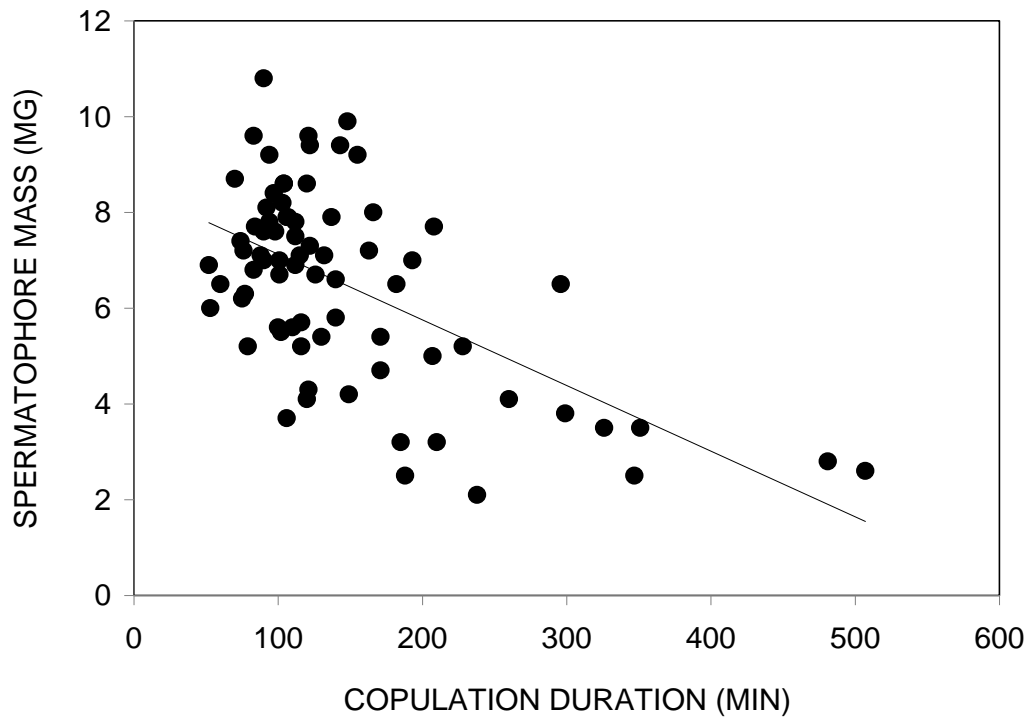


Figure 3. The negative relationship between the mass of the spermatophore produced during copulation and the duration of copulation ($r = -0.596$, 74 df, $p < 10^{-7}$).